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The Amino Acid Sequence of Satyr Tragopan Lysozyme and Its Activity

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The amino acid sequence of satyr tragopan lysozyme and its activity was analyzed. Carboxymethylated lysozyme was digested with trypsin and the resulting peptides were sequenced. The established amino acid sequence had three amino acid substitutions at positions 103 (Asn to Ser), 106 (Ser to Asn), and 121 (His to Gln) comparing with Temminck's tragopan lysozyme and five amino acid substitutions at positions 3 (Phe to Tyr), 15 (His to Leu), 41 (Gln to His), 101 (Asp to Gly) and 103 (Asn to Ser) with chicken lysozyme. The time course analysis using N-acetylglucosamine pentamer as a substrate showed a decrease of binding free energy change, 1.1 kcal/mol at subsite A and 0.2 kcal/mol at subsite B, between satyr tragopan and chicken lysozymes. This was assumed to be responsible for the amino acid substitutions at subsite A-B at position 101 (Asp to Gly), however another substitution at position 103 (Asn to Ser) considered not to affect the change of the substrate binding affinity by the observation of identical time course of satyr tragopan lysozyme with turkey and Temminck's tragopan lysozymes that carried the identical amino acids with chicken lysozyme at this position. These results indicate that the observed decrease of binding free energy change at subsites A-B of satyr tragopan lysozyme was responsible for the amino acid substitution at position 101 (Asp to Gly).

Key words: lysozyme; amino acid sequence; egg white

Lysozyme is one of the best-characterized hydrolases, which cleave β-1,4 linkages of N-acetylglucosamine (GlcNAc) homopolymer and GlcNAc-N-acetylmuramic acid heteropolymer, which causes lysis of the bacteria containing that polymer in their cell walls. This enzyme is classified into three types, chicken type,[1] goose type,[2] and phage type.[3] The amino acid sequences of lysozymes have been reported for many birds, mammals,[4] fishes,[5] and insects.[6,7] Only some birds have goose-type lysozyme.[8] The others have chicken-type ones.

One of the most intensive investigations for amino acid sequences of chicken type lysozyme is for avian egg white, especially for Galliformes. The amino acid sequences of phasianid birds are known for 14 lysozymes and we established a rapid sequence determination method for chicken-type lysozymes from Galliformes.[9–15] This method enables us to detect the amino acid substitutions occurred in closely related mutants by peptide map fitting and quantitative amino acid analysis.

By the extensive study of chicken lysozyme, it is revealed that this enzyme carries six substrate binding sites called subsites A, B, C, D, E, and F. A substrate bound at the subsites is cleaved between subsites D and E through the conventional acid catalytic reaction of Glu35 and Asp52. This enzyme also has high transglycosylation activity in addition to the hydrolysis. However the reaction mechanism for transglycosylation on this molecule remains unknown.

In the previous studies, we found that limited substitution at subsites affects the substrate binding or reaction mechanisms such as hydration and transglycosylation. The time-course analysis of the degradation of a substrate, N-acetylglucosamine pentamer $(GlcNAc)_5$, with the HPLC measurement can be used to evaluate the differences of the binding free energy change at each subsite and rate constants for hydration and transglycosylation by computer simulation. Therefore we can directly estimate the contribution of the amino acid that occurs in the active site compared with the amino acid sequences of two mutant enzymes, which carry amino acid substitutions at the active site. The naturally occurring lysozyme molecules genetically related closely to chicken lysozyme which carry limited amino acid substitutions at substrate binding site, are useful to evaluate the reaction mechanisms of lysozyme such as substrate binding and the lysozyme catalysis mechanism, and the information obtained is applicable to the mutagenesis study focused on the amino acids that participate in the substrate binding and catalysis.

In this study we report the amino acid sequence of satyr tragopan (*Tragopan satyra*) lysozyme and compare its amino acid sequence and activity with

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other lysozymes to evaluate the enzymatic properties of this lysozyme.

Materials and Methods

Purification of lysozyme. Freshly laid satyr tragopan eggs were a kindly gift from Kobe Oji Zoo, Kobe, Japan and Temminck's tragopan eggs were from Kyoto Municipal Zoo, Kyoto, Japan. The egg white (33 ml) was extracted with 66 ml of water and treated with isoelectric precipitations at pHs 4.0 with 1 M HCl and 7.0 with 1 M NaOH and the clarified solution after centrifuge at 12,000 rpm was then put on a CM-Toyopearl column (1.5×46 cm) equilibrated with 0.03 M phosphate buffer (pH 7.0) with the flow rate of 20 ml/h and 5 ml each in volume was collected. The column was then eluted stepwise with the same buffer containing 0.3 M NaCl. The lysozyme fraction was rechromatographed on the same column with a gradient of 0.1 M to 0.3 M NaCl in the same buffer and with the same conditions. Enzyme activity was monitored by a lytic activity using the lyophilized cell wall of *Micrococcus luteus* (Sigma Co., USA) as a substrate. Lysozyme solution (10 to 100 μl) was added to the substrate suspension adjusted to OD 1.0 at 540 nm in 3.0 ml of 0.1 M phosphate buffer, pH 7.0, and monitored the reduction of absorbance at 540 nm.

Carboxymethylation. Lysozyme was reduced and carboxymethylated for structural analysis.[16] Namely, 10 mg of lysozyme was dissolved in 1.4 M tris-HCl buffer, pH 8.6, and then 1.2 g of urea, 100 μl of 5% EDTA, and 33 μl of β-mercaptoethanol were added. The solution was left for 1 h at 37°C under N_2 gas. After the reduction, 89 mg of monoiodoacetic acid in 300 μl of 1.0 M NaOH was added and left for 1 h at room temperature in the dark. The reaction mixture was desalted through Sephadex G-50 column (1.7×46 cm) in 0.2 M NH_4OH and protein fraction was lyophilized (Cm-Lysozyme).

Enzymatic Digestion. Cm-lysozyme (5 mg) was dissolved in 1 ml of tris-HCl buffer, pH 8.0, digested with trypsin (1/50, w/w, TR-TPCK, Cooper Biomedical Co., USA) at 37°C for 4 h. The digest was directly separated by HPLC.

Peptide Mapping and Sequence Analysis. The tryptic peptides were separated with a reversed-phase (RP) HPLC column (ODS 120A S5, 4.0×250 mm, Yamamura Chemical Co., Japan) using the JASCO 800 series HPLC (Japan Spectroscopic Co., Japan). The peptide elution was done with a linear gradient elution system of 0.1% trifluoroacetic acid (solvent A) and 60% acetonitrile in solvent A (solvent B). A gradient of 0% to 50% of solvent B was used for 130 min. Tryptic peptides were hydrolyzed in evacuated sealed tubes at 110°C for 20 hr with constant boiling HCl containing 0.05% β-mercaptoethanol. The resulting hydrolysates were analyzed with an amino acid analyzer (Model 835, Hitachi Co., Japan). The amino acids of tryptic peptides were sequenced by a protein sequencer model PSQ1 (Shimadzu Co., Japan).

Enzyme action.

a) Lysis activity. The lytic activity was assayed using the lyophilized cell wall of *M. luteus* as a substrate. Samples (10 to 100 μl) of the eluent were added to 3 ml of the substrate suspension in 0.1 M phosphate buffer, pH 7.0, adjusted to OD 1.0 at 540 nm. One enzyme unit was defined as the amount causing a decrease of 0.1 absorbance unit at 540 nm in the reaction for 1 min at 25°C.

b) Time course. The enzyme reaction was done with the substrate GlcNAc pentamer. The reaction mixture containing 0.1 mM lysozyme and 1 mM of substrate dissolved in 10 mM sodium acetate, pH 5.0 was incubated at 50°C. At a given reaction time, 500 μl of the reaction mixture was withdrawn and rapidly chilled in an ultra filtration filter (Ultrafree C3-LGC, Millipore Co.) containing 70 mg of Bio-Rex 70 in a cool cup (−80°C). The reaction mixture was then centrifuged through a filter membrane and the resulting solution was lyophilized. The dried sample was dissolved in 50 μl of water and 10 μl of the solution was put onto a gel filtration column (TSKgel G-Oligo-PW, Tosoh Co.) using a JASCO 800-Series HPLC. Elution was done with distilled water at room temperature. Each chitooligosaccharide was monitored and measured at 220 nm using the standard curve obtained from authentic saccharide solutions.

c) Computer simulation. The rate equation and binding free energy of the lysozyme-catalyzed reaction on the initial substrate GlcNAc pentamer was numerically solved to obtain the calculated time courses by the method previously reported. The rate constants, k_{+1}, k_{-1}, and k_{+2}, used in the calculation are shown in Fig. 1.[17,18]

Result and Discussion

Satyr tragopan lysozyme was purified by conventional purification method. A water extract of egg white was treated with acidic and neutral pHs to precipitate acidic and neutral proteins and the clarified solution was then chromatographed on a cation exchange column, which yielded a single peak with lytic activity (data not shown). The enzyme was then carboxymethylated and digested with trypsin. The digest analyzed by peptide mapping. The HPLC peptide map of satyr tragopan lysozyme was compared with that of chicken lysozyme (Fig. 2). The map-fitting method indicates that the change of

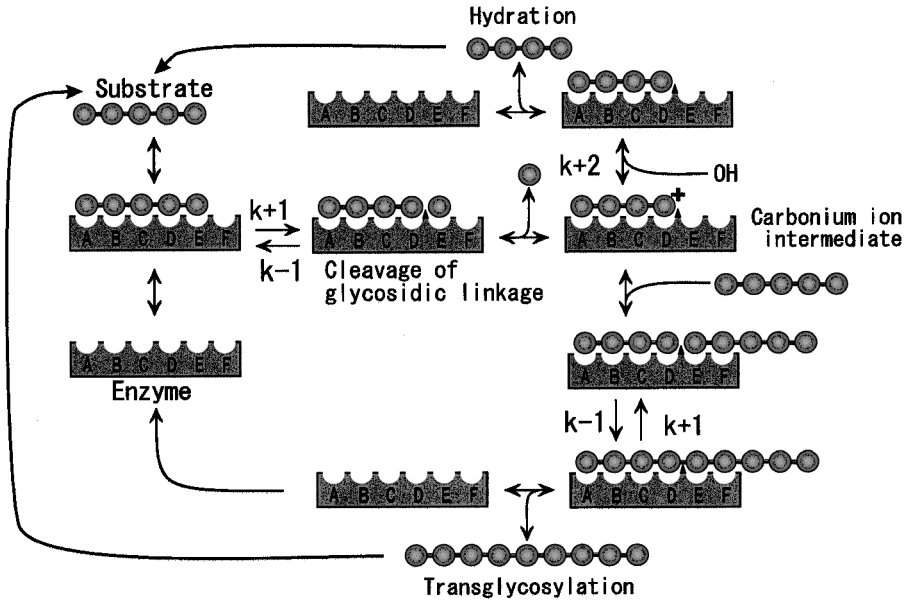

Fig. 1. Reaction Scheme for the Lysozyme-catalyzed Reaction of GlcNAc Pentamer.
In this scheme, k_{+1}, k_{-1}, and k_{+2} are the rate constants for cleavage of the glycosidic linkage, transglycosylation, and hydration, respectively.

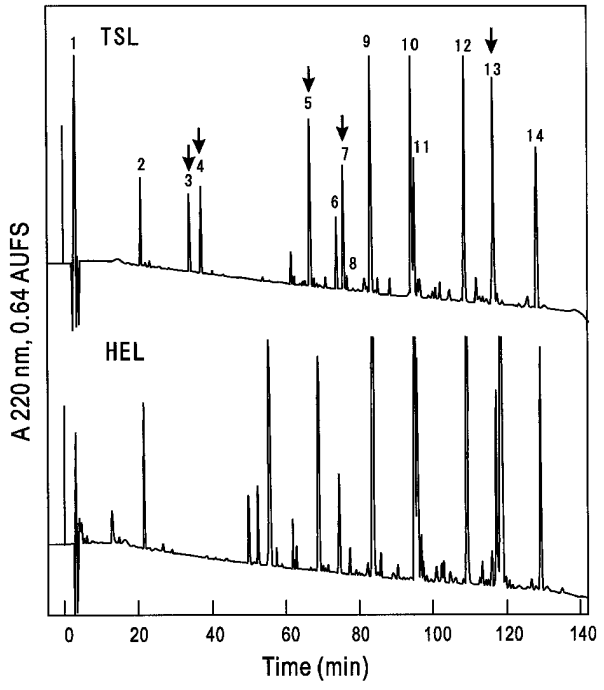

Fig. 2. Comparison of Reversed-phase HPLC Pattern of Satyr Tragopan Lysozyme with That of Chicken Lysozyme.
Peaks in satyr tragopan lysozyme that are indicated by arrows are the peaks appearing in different positions and with different amino acid compositions when compared with chicken lysozyme. For detailed conditions of HPLC see the text. HEL, chicken lysozyme; TSL, satyr tragopan lysozyme.

retention time of peptide peaks and the subsequent amino acid analysis of their amino acid compositions reveals the amino acid substitution, deletion, or insertion in a tryptic peptide. Further, the insertion or deletion between tryptic peptides including Lys or Arg can be detected by the amino acid analysis stoichiometrically as well as the identification a free Lys or Arg residue derived from successive Lys-Arg or Lys-Lys residues. In case of undetectable amino acid substitution by amino acid analysis such as Asn/Asp or Gln/Glu, it has been proved to be detectable by the change of retention time. Therefore the amino acid compositions of all peaks of satyr tragopan lysozyme were analyzed. The peptides in each peak of satyr tragopan lysozyme that have both the identical amino acid composition and the identical elution position to chicken lysozyme were considered to have the identical amino acid sequence as described above. The peptides that have different amino acid compositions and elution positions were analyzed for their amino acid sequences, and substitutions were found to occur on the peptides in peaks 3, 4, 5, 7, and 13 by this analysis (indicated by vertical arrows in Fig. 2). The result proved that these peptides have substitutions at Phe3 to Tyr, His15 to Leu, Gln41 to His, Asp101 to Gly and Asn103 to Ser when compared with the corresponding tryptic peptide of chicken lysozyme (Fig. 3).

The peak correspond to T13 was obtained as a single peak on the peptide map of satyr tragopan lysozyme. On the map of chicken lysozyme, T13 normally shows the multiple peaks that are caused by the deamidation of Asn103 in T13 peptide. This result may be due to the amino acid substitution of

Asn103 in satyr tragopan lysozyme (Asn103 to Ser).

The amino acid sequence established for satyr tragopan lysozyme was compared with chicken, turkey, and Temminck's tragopan lysozymes[15] and the amino acid substitutions, compared to chicken lysozyme, probed by sequence analysis of these peptides, are boxed in on Fig. 3. This enzyme had 5, 4, and 3 amino acid substitutions when compared with chicken, turkey, and Temminck's tragopan lysozyme, respectively. Among these substitutions, Gly101 and Ser103 were located at the loop region of subsites A-C (Fig. 4). Asp101 is reported to interact with the substrate (GlcNAc at subsites A and B) by hydrogen bonding and the substitution by Gly at this position may cause the loss of hydrogen bonding. On Fig. 4, the possible conformation change of the side chain of amino acids at position 101 and 103 on chicken lysozyme were drawn by the energy minimization program on a Swiss PDB Viewer.[20] The result indicates that the change of the size and the location of the side chain after mutation showed the possibility of the loss of hydrogen bonding at subsites A and B (this simulation did not consider the main chain movements). The substitution at position 101 is also found in turkey and Temminck's tragopan lysozymes and the effect of amino acid substitution for enzyme activity is notable point. Further Ser103 was first found in phasianid bird's lysozymes. Although the importance of the contribution of the amino acid at position 101 is well known, limited information is available on the amino acid at position 103, on the loop region at the substrate binding subsites A-B.

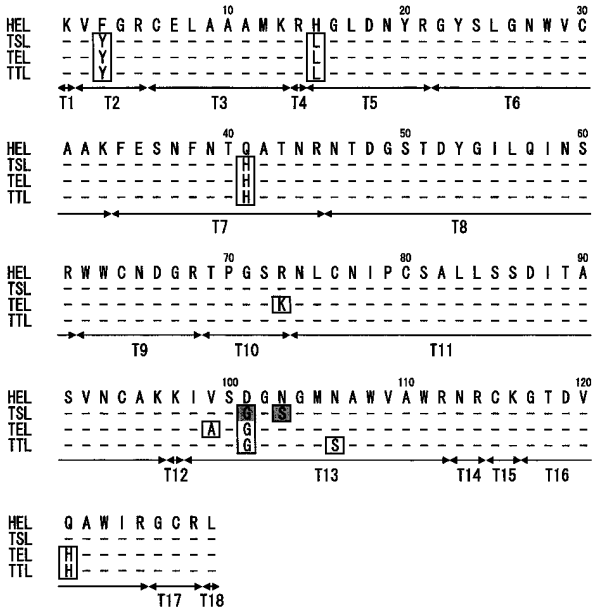

Fig. 3. Amino Acid Sequence of Satyr Tragopan Lysozyme and Its Comparison with Chicken, Turkey, and Temminck's Tragopan Lysozymes.

The peptides obtained are indicated by T numbers by Canfield's nomenclature of tryptic peptides of chicken lysozyme.[1] Substituted amino acids are boxed in. Other positions that contain no substituted amino acid are indicated by dashes. A substitution that occurred in satyr tragopan on the substrate binding site is highlighted. HEL, chicken lysozyme; TSL, satyr tragopan lysozyme; TEL, turkey lysozyme; TTL, Temminck's tragopan lysozyme.

As chicken-type lysozyme catalyzes not only hydrolysis of sugar chains but also a transglycosylation reaction, the product of oligosaccharide shows complicated patterns. However, the time course analysis using GlcNAc pentamer allowed the evaluation of the effects of amino acids substituted at the subsites, by computer simulation analysis (Fig. 1). To evaluate the contribution of amino acid substitutions to substrate binding and lysozyme catalyzed reaction, the time course of satyr tragopan lysozyme for the GlcNAc pentamer was analyzed and is shown in Fig. 5 with time courses of chicken, turkey, and Temminck's tragopan lysozymes. In the pattern of satyr tragopan lysozyme, the order of the concentration of product oligomer at 20 min of reaction was 2, 1, 3, 4, and 5 instead of 1, 2, 4, 3, and 5 for chicken lysozyme. This pattern is found to be similar to that of turkey and Temminck's tragopan lysozymes (Fig. 5). These lysozymes have the same substitution

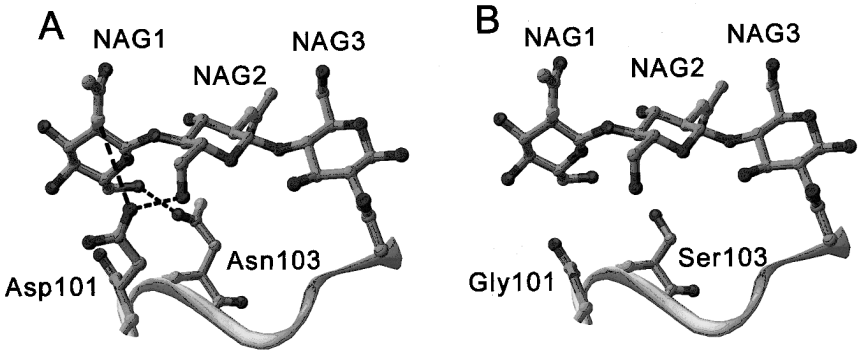

Fig. 4. Tertiary Structure of the Region of Subsites A-C.

The loop region (Asp101 to Trp108) is drawn by a ribbon diagram and the substrate GlcNAc trimer is drawn by ball and stick model. NAG1, NAG2, and NAG3 indicate the GlcNAc residues located at subsites A, B, and C, respectively. The model was prepared using the PDB data of 1HEW[19] with the program of Swiss PDB Viewer. A: chicken lysozyme, B: mutation model of Asn103Ser.

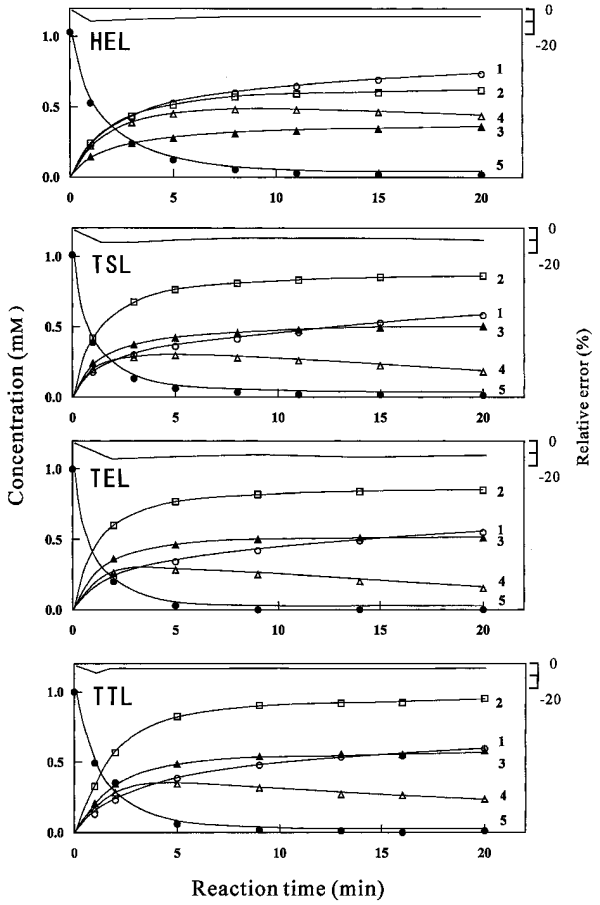

Fig. 5. Experimental Time Course for Chicken, Satyl Tragopan, Turkey, and Temminck's Tragopan Lysozymes.

HEL, chicken lysozyme; TSL, satyr tragopan lysozyme; TEL, turkey lysozyme; TTL, Temminck's tragopan lysozyme. The numerals in the figure indicate the polymerization degree of GlcNAc.

at position 101 as satyr tragopan lysozyme (Fig. 3). When compared with these lysozymes, satyr tragopan lysozyme has additional amino acid substitution (Asn103 to Ser) at the loop region at subsite A-C (Fig. 4). Taking the above factors into consideration, we estimate that the amino acid substitution at position 101 affects the difference of reaction patterns between satyr tragopan and chicken lysozyme, but the substitution found only on satyr tragopan lysozyme at position 103 do not affect the substrate binding and cleavage by the comparison of the time course patterns.

To confirm this notion, experimental time courses were then simulated by computer analysis. The values of binding free energy and the rate constants, k_{+1}, k_{-1}, and k_{+2} were obtained by the calculated time course fitted to the experimental ones (patterns not shown). The values of binding free energy and the rate constant for calculated time courses for these lysozymes are listed in Table 1. The difference of the values of binding free energy were found at subsites A and B. Namely, the binding free energy of subsites

Table 1. Estimated Reaction Parameter Values for HEL and TSL

	Binding free energy (kcal/mol)						Rate constant (s^{-1})		
	A	B	C	D	E	F	k_{+1}	k_{-1}	k_{+2}
HEL	−2.0	−3.0	−5.0	4.5	−2.5	−1.5	0.93	40.0	0.30
TSL	−0.9	−2.7	−5.0	4.5	−2.5	−1.5	0.93	40.0	0.30

k_{+1}, k_{-1}, and k_{+2} are the rate constants for cleavage of the glycosidic linkage, transglycosylation, and hydration, respectively.

A and B for satyr tragopan lysozyme were observed as −1.0 and −2.8 kcal/mol, respectively. Whereas, the values of subsites A and B for chicken lysozyme were −2.0 and −3.0 kcal/mol, respectively. The amino acid substitutions at the loop region of sites A-C would have a profound effect on the decrease in the affinity of subsites A and B for the sugar residues. As the Asp101 of chicken lysozyme forms hydrogen bonds with GlcNAc at subsite A and GlcNAc at subsite B, the substitution of Asp to Gly, which has no side chain at this position, will cause the loss of hydrogen bonding (Fig. 4). Therefore, the difference between the value of the binding free energy for satyr tragopan lysozyme and chicken lysozyme may be attributed to this substitution at subsite A (Asp101Gly).

The contribution of Asp101 in chicken lysozyme to the substrate binding at subsite A was reported to correspond to a binding free energy of about 30% of the total binding free energy of subsite A.[21] However, the substitution of Asp to Gly that causes the loss of the hydrogen bonding proved the contribution of Asp101 to be about 50% of the total binding free energy of subsite A. Further, the decreasing value of binding free energy were found at subsites A and B instead of subsites A and C as reported for Asp101-Glucosamine or Asp101-ethanolamine modified lysozyme.[21] The difference of the binding free energy for these subsites may due to the introduced chemicals and the removal of the side chain of this residue.

Another substitution at subsite B (Asn103Ser) seemed not to affect the change of the substrate binding affinity with the observation of similar time courses of satyr tragopan lysozyme to turkey lysozyme and Temminck's tragopan lysozymes, which have the identical amino acid at position 103 with chicken lysozyme (Fig. 5). Asn103 is reported to have hydrogen bonding with GlcNAc at subsite A. The result showed that the loss of the hydrogen bonding at position 103 caused by the substitution from Asn to Ser did not affect the substrate binding. It is understandable, therefore, that the contribution of the amino acid at position 103 to the substrate binding would be quite little. This is supported by the observation of similar time courses for native and

deamidated chicken or reeve's pheasant lysozymes at position 103 (Asn to Asp) frequently occurring for storage of the lysozyme (data not shown).[12] Combining these results, we conclude that the binding free energy change of satyr tragopan lysozyme observed at subsites A and B is caused by the amino acid substitution of Asp to Gly at position 101 on the loop region.

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